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Phase I Trial of 2B1, a Bispecific Monoclonal Antibody Targeting c-erbB-2 and FcγRIII[1]

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ABSTRACT

2B1 is a hispecific murine monoclonal antibody (BsMAh) with specificity for the c-erbB-2 and FcγRIII extraceiiular domains. This BsMAb promotes the targeted lysis of malignant cells overexpressing the c-erbB-2 gene product of the *HER2/neu* proto-oncogene by human natural killer cells and mononuclear phagocytes expressing the FcγRIII A isoform. In a Phase 1 clinical trial of 2B1, 15 patients with c-erhB-2-overexpressing tumors were treated with 1 h i.v. infusions of 2B1 on days 1, 4, 5, 6, 7, and 8 of a single course of treatment. Three patients were treated with daily doses of 1.0 mg/m^2, while six patients each were treated with 2.5 mg/m^2 and 5.0 mg/m^2, respectively. The principal non-dose-limiting transient toxicities were fevers, rigors, nausea, vomiting, and ieukopenia. Thrombocytopenia was dose limiting at the 5.0 mg/m^2 dose level in two patients who had received extensive prior myelosuppressive chemotherapy. Murine antibody was detectahle in serum following 2B1 administration, and lts hispecific hinding properties were retained. The pharmacokinetics of this murine antibody were variahle and best described hy nonlinear kinetics with an average $t_{1/2}$ of 20 h. Murine antibody bound extensiveiy to ali neutrophils and to a proportion of monocytes and lymphocytes. The initial 2B1 treatment induced more than 100-foid increases in circulating ievels of tumor necrosis factor-α, interieukin 6, and interieukin 8 and iesser rises in granulocyte-monocyte colony-stimuiating factor and IFN-γ. Brisk human anti-mouse antibody responses were induced in 14 of 15 patients. Several minor clinical responses were observed, with reductions in the thickness of chest waii disease in one patient with disseminated breast cancer. Resolution of pieurai effusions and ascites, respectively, were noted in two patients with metastatic colon cancer, and one of two liver metastases resolved in a patient with metastatic colon cancer. Treatment with 2B1 BsMAb has potent immunological consequences. The maximum tolerated dose and Phase 11 dally dose for patients with extensive prior myelosuppressive chemotherapy was 2.5 mg/m^2. Continued dose escalation is required to identify the maximally toierated dose for patients who have been less heavily pretreated.

INTRODUCTION

Since the introduction of monoclonal antibody technology, a variety of such antibodies has been used in clinical trials with the intent of inducing inflammation at tumor sites by recruitment of cytotoxic effector cells, thus exploiting the phenomenon of ADCC[3] (1–4). Although occasional clinical responses have been observed in patients treated with unconjugated mAbs (5), the induction of inflammatory infiltrates composed of cells bearing Fcγ receptors for IgG and possessing ADCC potential has not been demonstrated following mAb therapy. Interference with *in vivo* ADCC due to host IgG competition for cellular Fcγ receptor occupancy (6–8) may be partially responsible for these observations.

Advances in antibody engineering have made it possible to construct targeting molecules designed to circumvent some of these obstacles. One approach is to create antibodies with dual specificity for tumor antigens and for triggering molecules on cytolytic effector cells such as T-cells (9), NK cells (6, 10), and mononuclear phagocytes (11). It is possible to create highly purified, divalent bispecific proteins by chemical conjugation techniques (7, 12), by the fusion of distinct hybridomas to create quadromas secreting bispecific antibodies (6, 9, 10), or by creating single-chain bispecifics (13). Such molecules have been shown to redirect the cytotoxic properties of the targeted effector cell population against antigen-expressing tumor cells, usually at far lower concentrations than their corresponding parental antibodies (6). Also, appropriately designed BsMAbs promote targeted cellular cytotoxicity in the presence of competing human IgG, thus negating the impact of Fcγ receptor occupancy (6, 11, 14). Thus, BsMAbs expand the repertoire of cellular effectors capable of executing antibody-promoted tumor lysis.

Several BsMAbs have been tested in human clinical trials. Most have been directed against tumor antigens and elements of the CD3/T-cell receptor complex. Nitta *et al.* (15) treated glioblastoma patients with intralesional IL-2-activated autologous leukocytes plus a BsMAb directed against tumor and CD3 and showed superior survival compared to similar treatment without the BsMAb. Bolhuis *et al.* (16) have treated ovarian cancer patients with i.p. infusions of activated T cells plus a BsMAb directed against the MOV-18 antigen and CD3, with encouraging preliminary response rates. Valone *et al.* (17) have treated breast cancer patients with a chemically conjugated BsMAb recognizing the c-erbB-2 product of the *HER2/neu* oncogene and FcγRI, the high affinity Fcγ receptor for monomeric immunoglobulin expressed by mononuclear phagocytes and IFN-activated neutrophils. In this report, we present results from a Phase I clinical trial of the BsMAb 2B1, which targets c-erbB-2 and an epitope on the extracellular domain of FcγRIII (CD16). FcγRIII is the low-affinity Fcγ receptor expressed by human natural killer cells (NK/LGL) and differentiated macrophages and contains transmembrane and cytoplasmic domains capable of activating these cells. Neutrophils (PMN) express the B isoform of FcγRIII, where the extracellular domain is anchored to the membrane by phosphatidylinositol glycan; binding to this FcγRIII isoform does not trigger PMN cytotoxicity (18). BsMAb targeting tumor and FcγRIII are of interest because they can provide tumor specificity for NK/LGL and differentiated macrophages, thus potentially harnessing and directing the cytotoxic properties of these cell populations.

The production and characterization of 2B1 have been described extensively (19–25). 2B1 binds to c-erbB-2 with $K_d = 3.0 \times 10^{-8}$ and to FcγRIII with $K_d = 5.6 \times 10^{-8}$. 2B1 efficiently promotes the *in vitro* lysis of c-erbB-2-expressing tumor cells by FcγRIII-bearing human NK/LGL or macrophages (19, 20, 21), with half-maximal lysis

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[3] The abbreviations used are: ADCC, antibody-dependent cellular cytotoxicity; NK, natural killer cells; BsMAb, bispecific mAb; LGL, large granular lymphocytes; PMN, polymorphonuclear lymphocytes; MTD, maximum tolerated dose; ECOG, Eastern Cooperative Oncology Group; HAMA, human antimouse antibody; MFI, mean fluorescence intensity; TNF, tumor necrosis factor; IL, interleukin; GM-CSF, granulocyte-macrophage colony-stimulating factor; ANC, absolute neutrophil count.

potentiation noted at 2B1 concentrations of less than 5 ng/ml, and corresponding *in vivo* antitumor effects.

The objectives of this Phase I trial were: (*a*) to identify the toxicity and maximally tolerated dose of 2B1 using a daily 1 h infusion schedule; (*b*) to examine the pharmacokinetics of i.v. infused 2B1; (*c*) to examine the biodistribution in peripheral blood of 2B1; and (*d*) to determine the immunogenicity of 2B1. A MTD has been identified for heavily pretreated patients, while dose escalation continues for patients without prior extensive myelosuppressive chemotherapy. The current report describes results for the first 15 patients on this study, whose outcomes contributed to the identification of the MTD for heavily pretreated patients.

MATERIALS AND METHODS

2B1

The design, production, and characterization of 2B1 have been described previously (19–23). Briefly, this BsMAb was prepared by fusing the murine hybridomas 520C9 and 3G8, which secrete IgG1 isotype mAbs binding to epitopes on the extracellular domains of c-erbB-2 (520C9) and FcγRIII (3G8). The resulting quadroma supernatant contained the BsMAb, which was purified by sequential ion exchange chromatography, to yield >99% pure, clinical grade reagent. Following the conduct of preclinical safety and toxicology studies, a Phase I trial was initiated under the investigator-sponsored investigational new drug application BBIND 4970 with approval of the Fox Chase Cancer Center Investigational Review Board. The clinical trial was initiated in May 1993.

Study Design

To be eligible for treatment, patients were required to be ≥17 years of age; they must have been diagnosed with advanced cancer for which no standard, effective therapy was available; they were required to have biopsy-proven adenocarcinoma with ≥30% of tumor cells overexpressing c-erbB-2 by immunohistochemistry; they were required to have ECOG performance status of 0 or 1; they could not have received chemotherapy or radiation therapy within 4 weeks of starting treatment with 2B1; and they must have recovered from prior treatment toxicity. Standard hematological parameters included a hemoglobin of ≥10 g/dl, WBC ≥3,000/mm^3 with PMN ≥1,000/mm^3, and a platelet count of at least ≥100,000/mm^3. Serum chemistry requirements included an aspartate transcarbamylase ≤2 times the upper limit of normal; a total bilirubin of ≤1.5 mg/dl, and a serum creatinine of ≤1.5 mg/dl. Patients were considered ineligible if they: had received prior murine immunoglobulin therapy; they had active brain metastases; were receiving concurrent exogenous corticosteroid therapy; had significant heart disease requiring antiarrhythmics, antianginal agents, or therapy for congestive heart failure; or had pulmonary dysfunction evidenced by an FEV_1 <1.0 liters or DLCO <50% of predicted. Once eligibility criteria were met, informed consent was obtained. Patients received a single course of 2B1 therapy. Cohorts of patients were assigned to treatment at daily doses of 1.0, 2.5, or 5.0 mg/m^2. Treatments were administered on days 1, 4, 5, 6, 7, and 8, initially in an inpatient setting. 2B1 was administered via 1 h intravenous infusion, after patients received lorazepam and meperidine as premedication. Patients were monitored for objective response 1 month after the initiation of therapy. Those with stable or responding disease were followed thereafter at monthly intervals. Multiple blood samples were obtained for pharmacokinetic analysis, flow cytometry, cytotoxicity assays, HAMA determinations, and circulating cytokine levels.

Laboratory Assessments

Immunohistochemistry. Representative sections of tumor from primary or metastatic sites were evaluated as described (26). For each patient sample, one formalin-fixed, paraffin-embedded, or frozen section was stained with supersensitive anti-c-erbB-2 (HER-2/neu) (CB11; Bio Genex, San Ramon, CA) and developed using a biotinylated anti-immunoglobulin antibody and an alkaline phosphatase-conjugated streptavidin detection system (Bio Genex). Slides were evaluated for percentage of tumor cells staining and for staining intensity. Patients were potentially eligible for therapy if 30% or more tumor cells stained positive based on visual estimation.

Pharmacokinetics. Serum samples were obtained in association with the initial infusion at multiple time points for pharmacokinetic determination. Blood was drawn at 5, 15, 30, and 45 min and at the completion of the 1-h infusion and then at 5, 15, 30, 45, and 60 min and 1.5, 2.0, 2.5, 3, 4, 8, 12, 24, and 48 h after infusion. A dual antibody ELISA-based assay was performed using these samples to determine circulating murine immunoglobulin concentrations. Briefly, 96-well, polystyrene medium-binding plates were coated with 10 μg/ml rat antimouse IgG (Jackson Immunoresearch, Willow Grove, PA) overnight at 4°C. PBS with 0.05% Tween 20 and 3% BSA was used to block nonspecific binding. Plates were blocked for 2 h at room temperature and washed; then varying known concentrations of 2B1 (0–400 ng/ml) were added to the standard wells to develop a standard curve for each assay. 1:10 and 1:50 dilutions of patient samples were added to test wells and incubated at room temperature for 2 h. The plates were washed three times with PBS, 0.05% Tween 20, 1% BSA, and incubated with peroxidase-conjugated rat anti-mouse IgG (1:5,000–1:10,000; Jackson Immunoresearch) for 1 h at room temperature. Antibody reactivity was detected by developing the color reaction using the substrate tetramethylbenzidine, and the plates were read on a standard plate reader at 450 nm. The concentration of murine immunoglobulin in serum then was extrapolated from the standard curve. Pharmacokinetic analysis of the data was determined using the LAGRAN program (27).

HAMA. HAMA was assayed using a modification of the above method, coating the plates with whole 2B1 IgG1, murine IgG1 (MOPC-21; Sigma Chemical Co.), as well as $F(ab')_2$ fragments of 520C9 and 3G8 to characterize the specificities of the anti-murine antibody response. Blocking was performed using PBS with 0.05% Tween 20 and 0.5% BSA. The test wells were incubated with 1:50 serum dilutions, while known concentrations of primate anti-mouse IgG (Immunomedics, Morris Plains, NJ) were added to standard wells to develop a standard curve for each assay. The secondary antibody was a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-human IgG (Boehringer Mannheim, Indianapolis, IN). Antibody reactivity was detected by developing the color reaction using the substrate *o*-phenylenediamine dihydrochloride and read at 490 nm. The serum concentration of HAMA then was extrapolated from the standard curve.

Specimen Procurement and Preparation. Forty to fifty ml of peripheral blood were collected in acid citrate dextrose tubes at baseline and at the end of infusion on day 1, pre-infusion on day 4, and at the end of infusion on day 8. Polymorphprep (GIBCO-BRL, Life Technologies, Inc., Grand Island, NY) was used for Ficoll density gradient separation to obtain total WBC containing granulocytes, lymphocytes, and monocytes. Residual RBC were hypotonically lysed using 0.2% PBS and equilibrated with 1.6% PBS. All cell suspensions were examined for viability using 0.4% trypan blue stain dye exclusion (GIBCO). Cell viabilities always exceeded >95%.

Culture Media and Cells. RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine sera (Intergen Company, Purchase, NY), 2 g/liter $NaHCO_3$, 6 g/liter HEPES, 100 units/500 ml insulin (Novo Nordisk, Bagsvaerd, Denmark), 5 mg gentamicin (GIBCO), 2500 units/500 ml penicillin/streptomycin (JRH Biosciences, Lenexa, KS), 146 mg/500 ml glutamine (Gibco; complete media) was used for all human cells and SK-OV-3 cells. The SK-OV-3 cell line derived from a human ovarian carcinoma was obtained from the American Type Culture Collection (Rockville, MD).

mAbs. Simultest control IgG1-FITC/IgG2a-PE, goat anti-mouse IgG GAM-FITC, (MY31) anti-Leu19 (CD56)-PE, (B73.1) anti-Leu11c (CD16)-PE, (MΦ-P9) anti-LeuM3 (CD14)-FITC, and (L243) anti-HLA-DR-PE were obtained from Becton Dickinson (San Jose, CA). 2B1 was supplied by Chiron Corporation and fluorescein conjugated using the Quick Tag FITC Conjugation Kit (Boehringer- Mannheim), according to the manufacturer's instructions.

Flow Cytometry. Cells ($1–5 \times 10^5$) were washed with PBS, incubated 30 min at 4°C with mAb, and then washed in PBS. Paraformaldehyde (1%) in PBS was used for fixation prior to analysis. Analysis was performed using a FACScan (Becton Dickinson) coupled to a Consort 30 computer (Hewlett Packard, San Francisco, CA). Flow data was collected on forward- and side-scattered gated populations. Flow data from 10,000 events were collected, and the expression of cell surface markers was analyzed by two-color contours. Data were evaluated by determining the MFI of the positive cell populations, based on the difference of the mean channels of fluorescence for the specific and control mAbs, and by determining the percentage of positive cells in each gated cell population (22).

Cytokine Levels. TNF-α, IL-2, GM-CSF, IFN-γ, and IL-1b determinations were performed on the blood samples obtained for pharmacokinetic analysis using immunoenzymetric assay kits (Medgenix Diagnostics SA, Fleurus, Belgium). Cytokine concentrations (pg/ml) were determined by interpolation of the standard curves for each cytokine.

IL-6, IL-8, and elastase were performed by Lucien Aarden (The Netherlands Red Cross, Amsterdam, the Netherlands) as described (28–30). IL-6 and IL-8 (pg/ml) were measured by ELISA; elastase (ng/ml) was measured by RIA for α1-anti-trypsin complexes.

Clinical Assessments

Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria as described (31). Objective response was evaluated using standard ECOG criteria. Minor responses were defined as diminution in overall tumor area, lasting at least 1 month, but without 50% or greater reductions.

Statistical Analysis

Student's *t* test was used to determine the significance of results among the treatment cohorts.

RESULTS

Tumor Screening. From April 1, 1993 to December 15, 1994, a total of 356 tumor samples were screened for overexpression of c-erbB-2 by immunohistochemistry. Overall, 111, or 31% of the samples, expressed sufficient c-erbB-2 (defined as expression in 30% or greater tumor cells in the analyzed specimen). Twenty-two % of screened gastrointestinal primary cancers overexpressed c-erbB-2, as did 30% of gynecological, 22% of lung, and 29% of renal cell adenocarcinomas. Although a minority of all screened samples overexpressed c-erbB-2, 63% of the screened primary breast cancer specimens were positive.

Patient Characteristics. Table 1 summarizes patient characteristics, dose level assignment, and cumulative 2B1 dose administered. A total of seven men and eight women were treated, all with ECOG performance status of 0 (n = 3) or 1 (n = 12). Ages ranged from 24 to 69, with a median age of 55 years. Total administered 2B1 doses ranged from 8.7–60 mg.

Toxicity. A variety of non-dose-limiting toxicities was routinely observed in treated patients. Typically, patients experienced fever, rigor, nausea, occasional vomiting, and diarrhea, commencing at the conclusion of the 1 h 2B1 infusion, and lasting up to 4 h. These symptoms were alleviated by the prophylactic administration of meperidine and lorazepam or by treatment with acetaminophen, prochlorperazine, and meperidine. Three patients experienced transient dyspnea and arterial oxygen desaturation that was self-limiting and rapidly responsive to nasal oxygen. One patient treated at dose level 3 experienced transient grade 3 hypotension responsive to i.v. fluids.

Table 1 *Patient characteristics*

I.D.	Age	Sex[a]	Primary	Daily dose of 2B1 (mg/m^2)	Cumulative 2B1 dose (mg)	Prior systemic regimens (#)
1	52	F	Ovary	1.0	9.2	7
2	69	F	Lung	1.0	10.1	0
3	66	F	Breast	1.0	10.6	9
4	64	M	Pancreas	2.5	27.0	2
5	58	M	Stomach	2.5	30.0	3
6	24	M	Rectum	2.5	29.7	1
7	48	M	Kidney	2.5	28.5	4
8	68	F	Breast	2.5	22.2	6
9	57	M	Prostate	2.5	30.0	4
10	59	M	Colon	5.0	60.0	2
11	39	F	Kidney	5.0	53.4	0
12	60	F	Ovary	5.0	8.7	5
13	57	M	Colon	5.0	60.0	1
14	68	F	Colon	5.0	48.0	1
15	41	F	Breast	5.0	10.5	3

[a] F, female; M, male.

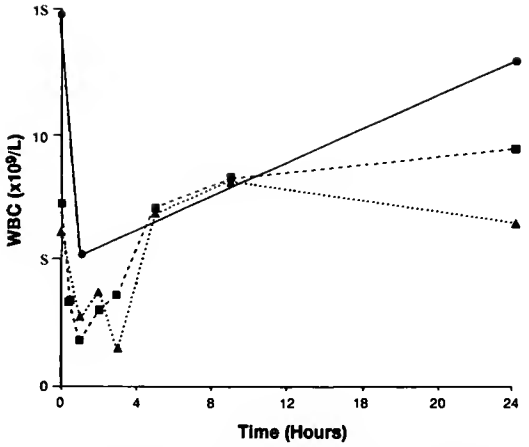

Fig. 1. Effects of 2B1 therapy on circulating WBC counts. Peripheral blood WBC were determined at the times depicted relative to the administration of the initial 1-h infusion of 2B1. The infusion commenced at time = 0. Mean WBC count results are shown for patients treated at dose level 1 (●), dose level 2 (▲), and dose level 3 (■).

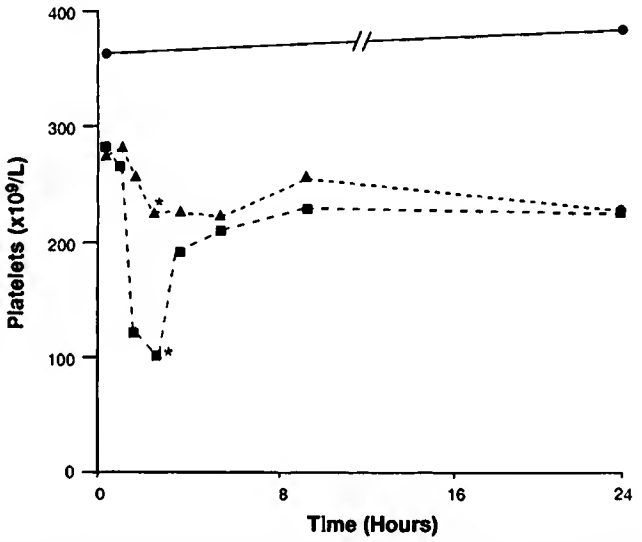

Fig. 2. Effects of 2B1 therapy on circulating platelet counts. Platelet counts were determined at the times depicted relative to the initial 1-h infusion of 2B1. The infusion commenced at time = 0. Mean platelet counts are shown for patients treated at dose level 1 (●), dose level 2 (▲), and dose level 3 (■). *, significant difference between dose levels 2 and 3 (P = 0.029).

The majority of patients experienced transient grades 3 and 4 leukopenia. This was primarily due to granulocytopenia, but monocytopenia and lymphopenia also were noted. The onset of leukopenia typically was noted 30 min into the initial 2B1 infusion, with nadirs persisting for 1–2 h, followed by recovery within 24 h (Fig. 1). Grade 3 toxicity was noted in 1 of 3 patients treated with 1.0 mg/m^2 (nadir ANC = 648), in 4 of 6 patients treated with 2.5 mg/m^2 (mean nadir ANC = 101), and in 5 of 6 patients treated with 5.0 mg/m^2 2B1 (mean nadir ANC = 154). The mean duration of neutropenia was approximately 6 h (2.5 mg/m^2) and 5 h (5.0 mg/m^2). No patients developed evidence of infections while they were neutropenic. Because this toxicity was transient and without infectious sequelae, leukopenia was not considered to be a dose-limiting toxicity of 2B1 treatment.

Platelet counts dropped significantly in patients treated at dose level 3, as shown in Fig. 2. The MTD was established by grades 3 and 4 thrombocytopenia, respectively, in two patients treated with 5.0 mg/m^2 2B1. Platelet count patterns were similar to those seen for

leukocytes, although nadirs tended to be more prolonged. At the lowest dose level, the mean platelet count nadir was 283,000/mm^3; at 2.5 mg/m^2, the mean nadir was 198,000/mm^3. At 5.0 mg/m^2, the mean nadir was 128,000/mm^3. At the conclusion of the initial 2B1 infusion, the mean platelet counts were 257,000 (± 30,000) in dose level 2 and 189,000 (± 75,000) in dose level 3 (P = 0.29); the mean baseline counts were virtually identical. The maximum loss of circulating platelets, taken as percentage (± SEM) of baseline values, was 23 ± 6, 29 ± 7, and 65 ± 10 for the 1.0, 2.5, and 5.0 mg/m^2 dose cohorts (P = 0.032 for comparison of 2.5 and 5.0 mg/m^2 dose levels). At the two higher dose levels, there was a clear association between the type and extent of prior therapy and treatment-induced thrombocytopenia. In the nine patients who had received either no chemotherapy (n = 3) or 5-fluorouracil-based chemotherapy (n = 6), the maximum loss of circulating platelets was 41 ± 7%, with mean and median platelet count nadirs of 196,000/mm^3 and 160,000/mm^3, respectively. The four patients in dose level 3 without prior marrow-toxic chemotherapy experienced an average maximum platelet count loss of 35.6%. In two patients with extensive prior cytotoxic chemotherapy, the maximum loss of circulating platelets was 89 ± 3% (P = 0.025 compared with the above results), and both the mean and median platelet count nadirs were 17,000/mm^3. The first patient was a 60-year-old woman with heavily pretreated metastatic ovarian carcinoma. Her baseline platelet count was 113,000/mm^3. Her platelet count rapidly nadired at 9,000/mm^3 and was associated with bleeding from venipuncture sites. She was treated with six units of platelets with recovery of her platelet count, and she received no additional 2B1 therapy. The second patient was a 41-year-old woman with heavily pretreated metastatic breast cancer. From a baseline of 181,000/mm^3, her platelet count nadired at 25,000/mm^3 4 h after the onset of the 2B1 infusion at 5.0 mg/m^2, and the platelet count did not recover to permit a second treatment for 5 days. She then was treated with 2.5 mg/m^2 2B1, and her platelet count declined from 163,000/mm^3 to 25,000/mm^3 with asymptomatic, associated hypotension requiring i.v. fluids.

Pharmacokinetic Analysis. The pharmacokinetic parameters based upon the serum concentration of murine 2B1 following the day-1 dose are summarized in Table 2. The system is best described by nonlinear kinetics with an overall mean (± SEM) plasma half-life of 20.0 ± 7.6 h. The mean (± SEM) volume of distribution was 11.6 ± 2.4 liters. Great interpatient variability was observed for all measured parameters; thus, no definitive correlation could be made between administered dose and pharmacokinetic parameters, including area under the curve.

Table 2 *2B1 pharmacokinetics*

Patient	Day-1 dose (mg/m^2)	Peak conc.[a] (ng/ml)	$t_{1/2}$ (h)	AUC[b] (ng/h/ml)	VD_{SS}[c] (liters)
1	1	195	10.4	1699	12.7
2	1	380	4.7	1245	5.5
3	1	245	121	25468	11.9
4	2.5	530	3.1	5036	4.4
5	2.5	790	20.5	22928	7.2
6	2.5	700	11.3	8199	10.3
7	2.5	1650	11.3	17219	5.7
8	2.5	1100	9.2	22613	3.4
9	2.5	1400	17.6	8940	13.7
10	5	1780	13.3	7159	22.4
11	5	530	12.4	11602	15.6
12	5	370	0.97	392	38.5
13	5	340	41.2	0.9	0.9
14	5	1700	19	15769	12.6
15	5	2050	10.3	11028	8.6
Mean			20 ± 7.6		11.6 ± 2.4

[a] Conc., concentration.
[b] Area under the curve.
[c] Volume of distribution at steady state.

Table 3 *Determination of circulating, functional 2B1*

Dose level	No. of Pts.	Baseline	2B1 concentration (ng/ml) at conclusion of infusion on Day 1	Day 8
1	3	0	18.0 ± 18.0[a,b]	32.3 ± 16.2
2	6	0	288.8 ± 71.8[b,c]	1088.8 ± 409.9
3	6	0	744.0 ± 131.5[c]	991.0 ± 219.5[d]

[a] Mean result ± SEM.
[b] P = 0.019 for comparison of dose levels 1 and 2.
[c] P = 0.029 for comparison of dose levels 2 and 3.
[d] Four samples obtained.

To determine the functional integrity of circulating 2B1, an ELISA was developed where a positive signal required that the antibody bind simultaneously to c-erbB-2 and FcγRIII extracellular domains. As shown in Table 3, there was a dose-related increase in circulating, functional 2B1, with mean levels of 744 ng/ml at the conclusion of the initial infusion of 5.0 mg/m^2. In patients treated at 2.5 mg/m^2/dose, circulating functional 2B1 levels rose nearly 4-fold by the conclusion of the day-8 infusion. The pattern of these results closely resembles that obtained when 2B1 concentrations in serum were determined based on the detection of murine IgG, as described above. Thus, circulating 2B1 retains bispecific binding properties after the i.v. administration of this BsMAb.

There was a clear association between circulating levels of 2B1 and leukocyte counts. As depicted in Fig. 3 for a representative patient treated at 2.5 mg/m^2, repeated daily 2B1 dosing led to sustained circulating 2B1 levels. With repeated dosing, the total WBC was progressively suppressed and remained so until circulating 2B1 levels dropped below 200 ng/ml.

2B1 Biodistribution in Peripheral Blood. Flow cytometry studies were performed to examine the binding of i.v.-administered 2B1 to circulating leukocytes. Murine antibody was detected consistently on neutrophils isolated from treated patients at the end of the initial 2B1 infusion, as detailed in Table 4. There was a trend (P = 0.054) for more murine antibody on neutrophils following treatment at the two higher dose levels, but no differences were noted between dose levels 2 and 3. All baseline and posttreatment patient samples were analyzed for available neutrophil-associated FcγRIII by incubating the cells with saturating concentrations of FITC-labeled 2B1 and comparing the shifts in MFI from simultest FL1 controls. A decline in the MFI shift in posttreatment samples was indicative of binding of administered 2B1 to the cells, and the degree of such inhibition was dose dependent. At the conclusion of the initial 2B1 infusion, the percentage of saturation of circulating neutrophils was 0.7% in dose level 1, 10% in dose level 2, and 59% in dose level 3. After the final infusion on day 8, the percentage of saturation increased to 15.3% in dose level 1, 72% in dose level 2, and 68% in dose level 3. Thus, treatment with multiple daily doses at 2.5 mg/m^2 was able to functionally saturate the available FcγRIII sites on circulating neutrophils. At baseline, 7.6 ± 1.3% of patients' circulating monocytes expressed expected levels of FcγRIII (31). Following therapy, the MFI shift due to murine antibody binding to monocytes via FcγRII was 22.0 ± 5.8% in five patients at dose level 2. We have shown previously that 2B1 primarily binds to human neutrophils by its 3G8 Fab interaction with FcγRIIIB, supplemented by Fc domain interactions with FcγRII.[4] Following 2B1 therapy, murine antibody deposition on NK cells was noted as well (not shown).

Induction of Secondary Cytokines. The toxicity syndrome induced by 2B1 therapy was reminiscent of symptoms associated with

[4] L. M. Weiner, R. K. Alpaugh, A. R. Amoroso, G. P. Adams, D. B. Ring, and M. W. Barth. Human neutrophil interactions of a bispecific monoclonal antibody targeting tumor and human FcγRIII, submitted for publication.

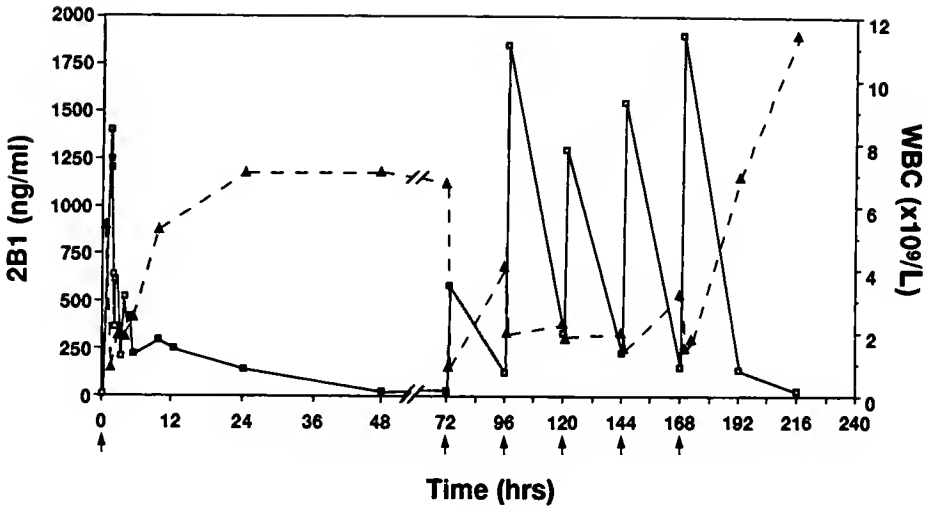

Fig. 3. Relationship of circulating murine antibody levels with WBC counts. In a representative patient treated at dose level 2 (2.5 mg/m^2/dose, with *arrows* indicating each infusion), circulating murine immunoglobulin levels (□) and WBC counts (▲) were serially determined.

cytokine therapy (32–34). Accordingly, serial patient blood samples were examined for the presence and concentration of a variety of cytokines. The 2B1 treatment-induced release of TNF-α was consistent and dramatic. As shown in Fig. 4, TNF-α levels averaged 19.2 pg/ml at baseline. Levels began to rise 30 min after initiating the 2B1 infusions and peaked at 2.3 h, gradually declining to near-baseline values over 24 h. More rapid TNF-α induction was noted in patients treated in dose level 3. Although the mean values at each time point suggest slightly higher values for dose level 3 patients, there were no statistically significant differences in peak TNF-α levels between dose level 2 (1938 ± 276 pg/ml) and dose level 3 (1486 ± 318 pg/ml). The mean time to peak TNF-α induction was not dose related. With subsequent doses, TNF-α induction was progressively less pronounced (data not shown).

Other circulating cytokine levels were induced by 2B1 therapy. IL-6, IL-8, and serum elastase levels were examined in patients 002, 004, and 009, while IFNγ, GM-CSF, IL-1β, and IL-2 levels were determined in patients 010–013. Greater than 30-fold elevations were noted in IL-6 and IL-8 levels. IFN-γ levels were less than 3.5 pg/ml in two patients at all times but rose to 100 and 125 pg/ml, respectively, in two other patients, with peak values noted 45 min after the end of the 1 h infusion of 2B1. IL-2 levels rose less than 2-fold from baseline in one patient, 4 h after the end of the 2B1 infusion, and IL-2 levels actually declined from baseline values in the other three samples. IL-1b levels did not rise above 0.4 pg/ml in any tested patient and were elevated at baseline in the other two patients. GM-CSF levels rose less than 2-fold in two of four tested patients, with peak levels noted 30 and 45 min after the conclusion of the initial 2B1 infusion. Elastase levels rose less than 10-fold in treated patients.

Table 4 *Binding of i.v. administered 2B1 to circulating neutrophils*

Dose level	No. of tested patients	ΔMFI[a]	% positive cells[b]
1	3	32.7 ± 7.3[d]	99.7 ± 0.3
2	5[c]	105.8 ± 26.1[d]	96.0 ± 2.5
3	6	74.5 ± 36.3	98.3 ± 1.7

[a] The difference of MFI of cells obtained at baseline and at the end of the initial 2B1 infusion. Analysis was restricted to cells in the neutrophil gate. No murine antibody was detected on the neutrophils in baseline samples. Baseline samples incubated with 2B1 exhibited a ΔMFI of 203 ± 44 (dose level 1), 138 ± 20 (dose level 2), and 115 ± 17 (dose level 3).

[b] The percentage of cells reactive with anti-murine antibody in the gated cell populations at the conclusion of the initial 2B1 infusion.

[c] In one sample, there were insufficient numbers of cells for interpretation at the end of the infusion.

[d] P = 0.054 for comparison of dose levels 1 and 2.

Induction of HAMA. Fourteen of 15 patients treated with murine 2B1 developed HAMA to the whole antibody (Table 5). The majority had circulating HAMA detectable by day 11 with peak values frequently seen beyond 3 to 4 weeks; the one nonresponder had no serum samples available for testing beyond day 7. The HAMA response was weak (<100 ng/ml) in three patients, moderate (100–2000 ng/ml) in four patients, and high (>2000 ng/ml) in seven patients. No obvious correlation could be demonstrated between doses of 2B1 administered and the degree of antibody response. All patients with high HAMA responses against the intact 2B1 IgG demonstrated the presence of an anti-murine antibody response directed against the F(ab')$_2$ fragments of the 2B1 parental antibodies 520C9 and 3G8.

Clinical Outcomes. One patient at the lowest dose level, with breast cancer metastatic to the chest wall, experienced a partial response lasting approximately 2 months. At dose level 3, three patients with metastatic colon carcinoma experienced minor responses, manifested by the resolution of ascites, pleural effusions, and one of two hepatic metastases, respectively, with stable measurable disease elsewhere. All of these minor responses lasted less than 3 months.

DISCUSSION

This is the first clinical evaluation of a BsMAb targeting a tumor antigen and FcγRIII. In contrast to observations with most previously tested murine mAbs, treatment with 2B1 led to a variety of toxicities, due in part to its ability to bind to FcγRIII. Several unconjugated murine antibodies have been shown to mediate toxicity distinct from hypersensitivity to murine proteins. Most notably, these have included R24, which induces complement fixation and pain at tumor sites (35), anti-GD2 ganglioside antibodies, which cause intense pain due to binding to peripheral nerve elements (36, 37), anti-CD3 antibodies causing an intense first-dose effect due to the release of TNF (32–34), and the anti-FcγRIII antibody 3G8, which caused bone pain in patients treated for immune thrombocytopenia (38) and produced a cytokine release syndrome in nonhuman primates (39). The toxicities observed in this trial most closely resemble those seen with anti-CD3 or 3G8 antibodies. Patients treated with OKT3 antibody for prophylaxis or treatment of organ allograft rejection commonly experience fever, rigors, dyspnea, tachycardia, nausea, vomiting, and diarrhea (40–43). Similarly, the treatment of humans (38) and primates (39) with 3G8 leads to granulocytopenia, the loss of circulating NK cells and the release of cytokines, with associated systemic symptoms. The structural determinants of these effects have not been fully defined but may include the requirements that the antibody target an activating

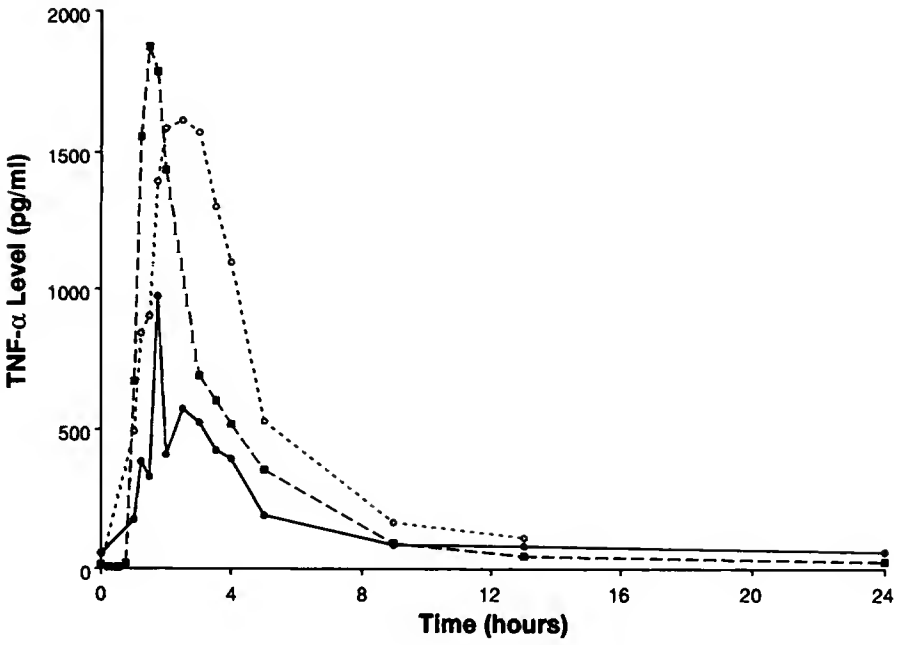

Fig. 4. Induction of circulating TNF-α levels by 2B1 therapy. TNF-α levels were determined as described in the text from serial blood samples obtained at various times relative to the administration of the initial 1-h infusion of 2B1. The infusion commenced at time = 0. Mean values are depicted separately for dose level 1 (●), dose level 2 (○), and dose level 3 (■).

molecule on leukocytes, that the binding to the molecule be divalent, and that the antibody Fc domain must be present to interact with macrophages and other Fcγ receptor-bearing cells. It was hypothesized that 2B1 would not trigger toxicity since it binds to FcγRIII-expressing leukocytes monovalently. Although 2B1 possesses an Fcγ domain, detailed *in vitro* studies have shown that approximately 80% of the binding of 2B1 to PMN is mediated solely by FcγRIII.[4] Also, the *in vitro* addition of 2B1 induces minimal but detectable complexing of neutrophils and mononuclear phagocytes (data not shown). The results described in this study clearly indicate that the monovalent binding of 2B1 to FcγRIII does not protect treated patients from toxicity caused by the binding of this BsMAb to cells bearing this Fcγ receptor.

The systemic administration of other bispecific antibodies containing Fcγ domains have led to similar toxicities (44). These toxicities have been minimal when effector cells were franked *ex vivo* with an anti-MOV18/CD3 bispecific IgG prior to their i.p. administration (16). However, systemic therapy with a chemically conjugated bispecific $F(ab')_2$ that binds to the c-erbB-2 epitope identical to that recognized by 2B1 and to an epitope on the extracellular domain of FcγRI has led to minimal toxicity in a single-dose range similar to those used in this study (17). This may be due to the absence of the Fcγ domain but also may result from the absence of FcγRI expression by human NK cells. Also, systemic toxicities and cytokine release have been observed following therapy with a BsMAb $F(ab')_2$ fragment (45). These findings suggest that the Fc domain may not account for all of the toxicities observed with 2B1 therapy.

Table 5 *Induction of human anti-mouse antibody*[a]

Patient no.	Cumulative dose (mg/m^2)	Peak human anti-mouse antibody (ng/ml) against 2B1	520C9 $F(ab')_2$	3G8 $F(ab')_2$
1	6	185	345	140
2	6	3,300	12,000	39,000
3	6	110	ND[b]	ND
4	15	3,000	19,500	>50,000
5	15	70	105	ND
6	15	65	80	ND
7	15	38,000	7,500	15,000
8	15	50	ND[b]	195
9	15	>50,000	7,000	>50,000
10	30	>50,000	15,500	>50,000
11	30	>50,000	22,500	14,500
12	5	ND[c]	ND[b]	ND
13	30	310	80	175
14	30	475	300	260
15	7.5	5,000	210	1,650

[a] Values calculated based on primate anti-murine antibody standards.
[b] ND, no detectable response.
[c] No patient serum samples were available beyond day 7.

Most of the toxicities observed in this study were easily managed. Leukopenia was nearly universal at doses exceeding 1.0 mg/m^2 but was transitory and unassociated with infections. Neutropenia was common and was presumed to be related to the binding of 2B1 to neutrophils, with subsequent clearance of the antibody-coated cells. The monocytopenia may have been caused by antibody Fc domain-FcγRII interactions, with subsequent monocyte activation, possibly on the basis of associated cytokine release.

Thrombocytopenia was the dose-limiting toxicity of 2B1 therapy and was most pronounced in patients who had received extensive prior bone marrow stem cell-toxic chemotherapy. The origin of the thrombocytopenia has not been determined. Although platelets express FcγRII, we could not demonstrate *in vitro* or *in vivo* that 2B1 binds to platelets or that 2B1 promotes the complexing of platelets and leukocytes (data not shown). It also is possible that the thrombocytopenia resulted from vascular endothelial activation following the induction of circulating cytokines by 2B1 therapy. Thrombocytopenia is commonly observed in sepsis and has been noted in clinical trials of cytokines such as TNF-α (46, 47). Following 2B1 therapy, the onset of thrombocytopenia coincided with the detection of rises in serum TNF-α levels.

Several aspects of the biodistribution of 2B1 are noteworthy. Unique pharmacodynamic considerations associated with 2B1 therapy likely contribute to the significant interpatient variability in the pharmacokinetics of this BsMAb. FcγRIII expression by circulating leukocytes provides an antigen reservoir sink for the circulating murine 2B1, thus leading to rapid clearance of the BsMAb. Although PMN comprise a competing cellular reservoir of FcγRIII, their presence did not preclude substantial circulating levels of intact, functional 2B1. This was predicted in part by *in vitro* studies demonstrating the rapid dissociation of 2B1 from PMN at 37°C. Since 2B1 rapidly dissociates from FcγRIII as the concentration of circulating BsMAb declines, it

is unlikely to provide NK cells and macrophages with stable c-erbB-2-targeting properties. Therefore, intact 2B1 must be able to diffuse to tumor sites and be present at concentrations of at least 1–5 ng/ml so its cytotoxicity potentiation properties can be clinically exploited (22). If the quantitative *in vivo* tumor targeting properties of 2B1, which have been described in animal models (23), reflect those seen with other clinically used murine antibodies (48), tumor retention of 0.001–0.1% injected dose/g is likely, and tumor concentrations of 50 ng/ml-5 μg/ml 2B1 may be anticipated following treatment with 5 mg/m^2 of 2B1. Such concentrations are capable of supporting maximal 2B1-promoted tumor lysis by human NK cells (22) and macrophages (21). Additional patients with serially biopsiable disease are being treated with 2B1 to examine murine antibody targeting to tumor sites.

The results of this clinical trial demonstrate that 2B1 interacts *in vivo* with FcγRIII-bearing leukocytes and that free circulating 2B1 is available for diffusion into tumor, probably at concentrations that are sufficient for the promotion of targeted tumor lysis, despite the relatively low MTD. It remains to be demonstrated that a sufficient number of potentially cytotoxic leukocytes migrate to or preferentially proliferate at tumor sites for this cytotoxicity to occur. However, the clinical outcomes observed in this trial suggest that tumor growth is modulated in some patients by therapy. Although this may be due to bispecific antibody-promoted tumor lysis, other mechanisms, such as the induction of multiple immunoregulatory and antiproliferative cytokines, cannot be excluded.

Retargeting the cytotoxicity of defined effector cell populations has been shown to exhibit substantial promise in a number of preclinical *in vitro* and *in vivo* (23, 49, 50) models. Although relatively few clinical trials of this concept have been performed thus far, nearly all have shown encouraging preliminary evidence of biological and clinical activity for BsMAb targeting the CD3/T-cell receptor complex (15, 16), FcγRI (17), and with this current report, FcγRIII. Further studies to understand the factors regulating the biological and clinical determinants of response should lead to the development of improved targeting molecules and treatment strategies.

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